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Original Article

Phytochemical screening and bioactivity studies of *Phyllanthus wightianus*

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ABSTRACT

This study was designed to determine the primary and secondary metabolites present from the leaves of *Phyllanthus wightianus* using various analytical techniques. Furthermore the antioxidant and anti-inflammatory activities of ethanolic extract of the leaves of *P. wightianus* were investigated using standard models. The results show that the leaves exhibit good antioxidant activity and protective to HRBC (Human Red Blood Cell) membrane due to the presence of some valuable phytochemicals present in the leaves.

1. Introduction

Phyllanthus wightianus Muell Arg – Synonyms – *Reidia floribunda* (Euphorbiaceae) is monocious sub shrub to 1 m branchless in lose spirals, pubescent. Leaves are alternate, distiches, elliptic to oblong, dark green above. Flowers are reddish, and fruits are pendulous through the year. Plant is distributed to Peninsula (Hook.f.l.c), Hills (750) 1000 m, on the floor and border of shoals and also available abundantly in local areas. The whole plant of *P. wightianus* has long been used as a constituent of an ethno-medicine for bone setting, as an antidiarrhoeal, against jaundice and for treating dieresis. Chemical constituents and in-vitro antioxidant activity of *P. wightianus* were reported. The whole plant extracts were subjected to isolation of their compounds of isomeric sterol mixture of (stigmasterol, compesterol and sitosterol), fredilin, lupeol, gallic acid, bergenin, geraniin, corilagin and ellagic acid were established through the use of column chromatographic methods. The percentage of tannins was also determined and estimated using the HPLC method.[1–3]

Plant extracts were investigated to estimate the primary and secondary metabolites using various analytical techniques and the alcoholic leaves extract subjected to bioactivity studies of in-vitro antioxidant and anti-inflammatory using standard assay like reducing power assay, hydrogen peroxide and (DPPH) α, α-diphenyl-β-picryl hydrazyl methods and in-vitro antiinflammatory studies through HRBC membrane stabilization in order to protect by using the plant extract of *P. wightianus*.

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2. Material and methods

2.1. Collection and identification of plant material

The leaves of *P. wightianus* were collected from the Javadi Hills, Vellore district, Tamil Nadu during December 2010. They were identified in Rapinat herbarium St. Joseph's College (Autonomous), Tiruchirappalli, Tamil Nadu, India.

2.2. Drugs and chemicals

DPPH was obtained from HiMedia laboratories Pvt. Ltd. Mumbai, India. All other chemicals used in this study were of analytical grade.

2.3. Determination of moisture and ash content

About 5 g of fresh leaves were taken in a pre-weighed silica crucible. It was kept in air oven for an hour at 110 °C. Then the weight of the dry leaves was found out. From the difference in weight, the amount of water was determined. The ash content was determined by incineration of the dry plant sample in muffle furnace at 400 °C.

2.4. Quantitative determination of primary metabolites

2.4.1. Preparation of sample solution

About 0.5 g of ash was digested with con. HCl and the whole was dissolved in water and filtered. The filtrate was made up to 100 mL in a standard flask. This made up solution was used for further analysis.

2.4.2. Determination of sodium, potassium and calcium

The standard sodium ion solution was prepared (0.586 g NaCl in 100 mL). From the above solution, nine different concentration (1.0, 1.5, 2.0… 5.0 mL) were prepared. These solutions were taken for flame photometric studies (Systronics mediflame 127). A standard graph was plotted by taking concentration of sodium on the X-axis and emission intensity shown by the flame photometric study on the Y-axis. Reading for the sample solution was fitted with the standard graph. The percentage of sodium in plant sample was determined. The concentration of potassium and calcium were also calculated by the same procedure. The standard potassium (0.750 g KCl in 100 mL) and calcium solutions (0.55 g $CaCl_2$ in 100 mL) were prepared.

2.4.3. Determination of iron and phosphorus

The determination of iron and phosphorous was done spectrocolorimetrically by standard graph method. The standard solutions of iron of different concentrations were prepared from the bulk solution (2.44 g of FAS in 250 mL). Each of the iron solution was treated with 4N HNO_3 and NH_4CNS. The percentage transmittance was measured at 470 nm. The nine different standard solutions of phosphorous were prepared from the bulk solution (0.1 g of KH_2PO_4 in 250 mL). Each of the phosphorus solution was treated with ammonium molybdate and ammonium vanadate. The percentage transmittance was measured.

2.4.4. Determination of sulfur

Sulfur was also determined by spectrocolorimeter method. Unlike other method, the sulfur in plant sample was converted into sulfate using $BaCl_2$.

2.4.5. Determination of copper, manganese and zinc

The concentration of copper, manganese and zinc in plants sample was determined by AAS (Atomic Absorption Spectrometer). A standard solution of Copper was prepared by dissolving 3.929 g of $CuSO_4.5H_2O$ in 1000 mL of water and 10 mL of the solution was diluted to 100 mL with water. Standard solutions of Mn (3.076 g of Manganese sulfate in 1000 mL, treated with Nitric acid:perchloric acid (9:1) and Zn (4.398 g of Zinc sulfate in 1000 mL) were prepared. The determination of Cu, Mn and Zn was done by using AAS with the specifications for mono element hollow cathode lamp. The exact specifications should be as per the particular instrument used.

2.4.6. Determination of magnesium

The standard solution of magnesium was prepared by dissolving 3.076 g of $MgSO_4$ in 1000 mL of deionized water. Ten mL of this solution was diluted to 100 mL (100 ppm of Mn). This solution was used as standard solution. The magnesium was estimated by titrimetric method using standard EDTA with Erio-chrome black-T indicator at pH10 using ammonia as a buffer.

2.4.7. Determination of vitamins

Vitamin B was determined spectrocolorimetrically with the reagent ferric sulfate and KCNS. Vitamin A was estimated spectrocolorimetrically using acidic antimony chloride reagent by the standard graph method.

2.5. Quantitative determination of secondary metabolites

The total flavonoid and phenolic contents were quantified by spectrophotometeric method using Folin's Ciocalteaus reagent. The other secondary metabolites such as alkaloids, tannins, lignins, glycosides, serpentines, terpenoids and saponins quantified by HPLC method and C18 general purpose column. The mobile phase consisted of solvent A (Methanol) and solvent B (0.5% (v/v) orthophosphoric acid in water). The data were interpreted by the Millenium Chromatography Manager V4.0 Software.[4–13]

2.6. In-vitro screening for antioxidant activity of leaf extract, preparation of sample solution

Fresh leaves were collected, shade dried and powdered mechanically. About 100 g of the powder were extracted with 1000 mL of 70% ethanol by hot percolation method using soxhlet extractor for 4 h. The extract obtained was evaporated at 45 °C to get a semi solid mass. The yield of ethanolic extract was found to be 40%. This extract was used for further studies.[14–18]

2.7. DPPH radical scavenging activity

To determine the DPPH assay of sample by Gyamfi et al., method, free radical scavenging potential of *P. wightianus* leaf

extracts was tested against a methanolic solution of DPPH (α, α-diphenyl-β-picryl hydrazyl). When antioxidants react with DPPH, the DPPH was converted to α, α-diphenyl-β-picryl hydrazine with a discoloration. The degree of discoloration indicates the scavenging potentials of the antioxidant extract. The change in the absorbance produced at 517 nm has been used as a measure of antioxidant activity. The change in absorbance of the samples was measured. Free radical scavenging activity was expressed as the inhibition percentage calculated using the formula.

2.7.1. Calculation

$$\text{Percentage of anti} - \text{radical activity} = [A - B/A] \times 100$$

where, 'A' is absorbance of control & 'B' is absorbance of sample.

2.8. Reducing power assay

To determine the reducing power assay of sample by Yildrim et al., 1 mL of leaf extract was mixed with phosphate buffer (2.5 mL 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and ferricchloride (0.5 mL, 0.1%) and absorbance measured at 700 nm. Increased absorbance of the reaction mixture indicates stronger reducing power. The activity was compared with ascorbic acid standard.

2.8.1. Calculation

$$\text{Percentage scavenging activity} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the control. A_{test} is the absorbance in the presence of the sample.

2.9. Hydrogen peroxide scavenging activity

To determine the hydrogen peroxide assay of sample by Umamaheswari and Chatterjee et al., method, hydrogen peroxide solution (2 mM/L) was prepared with standard phosphate buffer (pH 7.4). Different concentration of the extracts in distilled water was added to 0.6 mL of hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The inhibition was calculated. Ascorbic acid was used as standard.

2.9.1. Calculation

$$\text{Percentage of } H_2O_2 \text{ radical scavenging activity} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

Where $A_{control}$ is the absorbance of the control. A_{test} is the absorbance in the presence of the sample.

2.10. In-vitro screening for anti-inflammatory activity of leaf extract

The HRBC membrane stabilization method was used to study the anti-inflammatory activity of sample extract. Human blood was purchased and mixed with equal volume of sterilized Alsever solution. Alsever solution contains dextrose, sodium citrate and sodium chloride in water.[19–23]

The blood was centrifuged and the packed cells were washed with isosaline and 10% v/v suspension was made with Isosaline. The drug samples were prepared by suspending the residues in hot water. The assay mixture contained the drug, 1 mL phosphate buffer; 2 mL hypo saline, 0.5 mL HRBC suspension and Dichlorofenac–Sodium 5 mg/mL was used as the reference drug. Instead of hypo saline 2 mL of distilled water was used in the control. All the assay mixture were incubated at 37 °C for 30 min and centrifuged. The hemoglobin content in the supernatant solution was estimated using spectrophotometer at 560 nm. The percentage hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water as 100%.

2.10.1. Calculation

The percentage of HRBC membrane stabilization was calculated using the formula,

$$\text{Percentage protection} = \frac{100 - \text{Optical density of drug treated sample}}{\text{Optical density of control}} \times 100$$

3. Results and discussion

The medicinal plants were analyzed to have the minerals potassium, sodium, calcium, magnesium, iron, phosphorus etc. The results of quantitative estimation of primary and secondary metabolites are given in Tables 1 and 2 respectively. The moisture and ash content were found to be 1.02% and 60% respectively. The highest percentage of iron and magnesium was noticed in the leaves of *P. wightianus*. Calcium was the most abundant macro element in the plants. It may be the plant acting as a bone setting for ethano medicine practices. The presence of zinc in the plant *P. wightianus* plays a major role as catalyst over 200 enzymes and capable of influencing immune system. Zinc maintains various reactions of the body which help to construct and maintain DNA, required for the growth and repair of body tissues. Phosphorus has a vital role in almost every chemical reaction within the body because it is present in every cell. It forms calcium phosphate with calcium in the bones & teeth in a 2:1 ratio. It is important in the utilization of carbohydrates, fats, and proteins for the growth and maintenance in the body. Phosphorous is estrogenic, immuno stimulant and anti-osteoporotic. The flavonoids are higher in the leaves and it may be attributed for the above studied for acting as good antioxidant and membrane stabilization. The plant was found to be a good source of Vitamin B6, which is involved in many aspects of macro-nutrient metabolism.

Table 1 – Micro & macro element analysis of leaves of *P. wightianus*.

Sl. No	Primary metabolite	Abundance
1.	Sodium (%)	0.58
2.	Potassium (%)	3.26
3.	Calcium (%)	4.58
4.	Iron (ppm)	56.69
5.	Phosphorus (%)	0.58
6.	Sulfur (%)	0.48
7.	Copper (ppm)	1.05
8.	Manganese (ppm)	12.36
9.	Zinc (ppm)	2.19
10.	Magnesium (%)	2.65
11.	Vitamin A (μg/kg)	0.02
12.	Vitamin B6 (μg/kg)	36.54

Accumulated evidence suggests that ROS can be scavenged through chemoprevention utilizing natural antioxidant compounds present in foods and medicinal plants. The antioxidant activity of *P. wightianus* leaf extract were studied on the following methods like DPPH, hydrogen peroxide, and reducing power scavenging activity. The study shows the inhibition percentage as 19.0%, 56.0%, and 64% respectively. The antioxidant activity of ethanolic extracts observed higher potential in reducing power assay.

The lysosomal enzymes released during inflammation produce a variety of disorders. The extra cellular activity of these enzymes is said to be related to acute or chronic inflammation. The non steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane. Since HRBC membrane are similar to lysosomal membrane components the prevention of hypotonicity induced HRBC membrane lysis is taken as a measure of anti-inflammatory activity of drugs. The results were reported in Table 3. It was observed that the ethanolic extract shows significant anti-inflammatory activity at the concentration of which is comparable to the reference standard drug Dichlorofenac–Sodium 5 mg/mL. The anti-inflammatory activity of the extracts were concentration dependent, with the increase in concentration, the activity is also increased. The ethanolic extract of *P. wightianus* has significant anti-inflammatory activity.

Table 2 – Estimation of secondary metabolites of leaves of *P. wightianus*.

Sl. No	Secondary metabolite	Abundance
1.	Total flavanoid (mg/kg)	1.02
2.	Phenols (mg/kg)	0.56
3.	Alkaloids (mg/kg)	0.82
4.	Tannin (mg/kg)	0.56
5.	Lignin (mg/kg)	0.42
6.	Glycosides (mg/kg)	0.08
7.	Serpentines (mg/kg)	0.06
8.	Terpenoids (mg/kg)	0.08
9.	Saponins (mg/kg)	0.02

Table 3 – *In-vitro* anti-inflammatory activity of *P. wightianus*.

Sl. No	Concentration of extract mg/mL	Percentage protection
1.	100	–54.7%
2.	200	0.57%
3.	300	123.20%

Control – without extract; Reference – Dichlorofenac–Sodium 5 mg/mL.

4. Conclusion

The interpretation of the results give some useful conclusion and this study therefore provide some biochemical basis for the ethno medicinal use of extracts from *P. wightianus* in the treatment and prevention of various incurable diseases. As rich source of phytochemicals, minerals and vitamins present in the leaf of the plant *P. wightianus* can be further studied to use as a key ingredient for some valuable drugs. Furthermore, it is concluded that the plant extract act as a good source of antioxidant and membrane stabilization due to phytochemicals present in the plant extract.

Conflicts of interest

All authors have none to declare.

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